Towards reconstitution of human RNase P protein subunits with human and bacterial RNase P RNAs

Research Thesis

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by

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DEDICATION

Dedicated to the Ekeke family for their support, love, and blessings throughout my undergraduate career.

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LIST OF ABBREVIATIONS

RNase P	Ribonuclease P
RNP	Ribonucleoprotein
RPP	RNase P Protein
RPR	RNase P RNA
Eco	*Escherichia coli*
Hsa	*Homo sapiens*
Mth	*Methanothermobacter thermautotrophicus*
Pfu	*Pyrococcus furiosus*
Mja	*Methanocaldococcus jannaschii*
GS	Guide Sequence
IPTG	Isopropyl-β-D-thio-galactoside
DTT	Dithiothreitol
LB media	Luria-Bertani media
PMSF	Phenylmethylsulfonyl fluoride
EDTA	Ethylenediaminetetraacetic acid

PEI	Polyethlyenimine
s	second(s)
min	minute(s)
h	hour(s)
pre-tRNA	Precursor tRNA
tRNA	Transfer RNA
Abs_{600}	Absorbance at 600 nm
SDS	Sodium dodecyl sulfate
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
°C	Degree Celsius

ABSTRACT

RNase P is an endoribonuclease that catalyzes the removal of 5′-leaders in precursor tRNAs (pre-tRNAs), and it functions in all three domains of life as a ribonucleoprotein (RNP). The holoenzymes consist of an essential, catalytic RNase P RNA subunit (RPR) and a variable number of RNase P protein (RPP) subunits depending on the source. Bacterial RNase P is comprised of one RPR and one RPP. In contrast, eukaryotic RNase P contains one RPR and up to 10 RPPs. Intermediate in complexity, archaeal RNase P consists of one RPR and up to 5 RPPs. Although archaeal and eukaryal RPRs display pre-tRNA processing without RPPs, their activity is 10^2 to 10^6-fold slower than that of bacterial RPR. Despite similarities in the structure of all RPRs, archaeal and eukaryal RPRs are more dependent on cognate RPPs for biological function compared to their bacterial cousin. Recently, we have successfully reconstituted archaeal RNase P *in vitro* and begun to dissect the roles of individual subunits. However, the multi-subunit eukaryotic RNase P has proven refractory in this regard and prevented a dissection of the functional interplay between RNA and protein subunits, which is vital to understand protein-aided RNA catalysis in this catalytic RNP. Based on insights from studies on archaeal RNase P, we recently purified several human RPPs as binary complexes (with appropriate RPP partners) to decrease the number of possibilities in the order of RPP additions during human RNase P assembly *in vitro*. This thesis reports on these ongoing purifications and efforts to initiate reconstitution of the 11-subunit human RNase P. In parallel, we also exploited the availability of purified recombinant human RPPs to investigate if any of them (alone or in combinations) aid bacterial RNase P RNA

catalysis. Such an undertaking also gains clinical significance given the successful use of bacterial RNase P-based customized ribozymes for selectively cleaving oncogenic and viral mRNAs in human cells.

CHAPTER 1

INTRODUCTION

1.1 RNase P

RNase P is a catalytic ribonucleoprotein (RNP) responsible for catalyzing the removal of the 5′-leader from precursor tRNAs (pre-tRNAs) in all three domains of life (Fig. 1). It consists of an essential RNase P RNA subunit (RPR), and a variable number of RNase P protein (RPP) subunits depending on the source: 1, ≤ 5, and ≤ 10 RPPs in bacteria, archaea, and eukarya, respectively (1-4; Table 1). Although the RPR subunit from all three domains of life can cleave pre-tRNA in the absence of its cognate proteins, the bacterial RPR is the most efficient under the *in vitro* conditions tested: it exhibits a k_{obs} value ~ 10 min^{-1} compared to 10^{-1} and 10^{-5} min^{-1} displayed by the archaeal and eukaryal counterparts (5-7). There is an inverse proportionality between the RPPs and RPR-alone activity, with eukaryal RNase P having the highest protein: RNA mass ratio (70%), in comparison to 50% in archaeal and 10% in bacterial RNase P (1-4). Despite the RPRs from all three domains of life possessing universally conserved sequence and structural elements that are likely to make up the active site, the functional dependence of RPRs on the respective RPPs is clearly different. Given that even the single bacterial RNase P protein subunit assists the cognate RPR's binding affinity for the pre-tRNA/Mg^{2+} and enhances the RPR's cleavage rate (1, 8-11), it is of interest to determine why multiple RPPs are needed for the biological function of archaeal and eukaryal RNase P, and how

they cooperate with the single RNA moiety to create functional RNPs. Recent findings on archaeal RNase P are discussed below to provide a context to the specific aims on eukaryotic RNase P, which form the mainstay of this thesis.

Archaeal RPRs resemble their bacterial cousin yet are associated with 5 RPPs that have eukaryotic homologs (POP5, RPP21, RPP29, RPP30, RPP38/L7Ae; Table 1; 4, 5, 12). Archaeal RNase P is thus an interesting mosaic. In the Gopalan laboratory, archaeal RNase P from *Methanococcus maripaludis* (Mma), *Methanothermobacter thermautotrophicus (Mth), Pyrococcus furiosus (Pfu), and Methanocaldococcus jannaschii (Mja)* have been successfully reconstituted *in vitro* using recombinant RPR and RPPs (5, 13-15). These biochemical studies revealed that four of the five archaeal RPPs function as binary complexes (RPP21•RPP29 and POP5•RPP30) and that they collectively increase the RPR's k_{cat}/K_m by two to three orders of magnitude (5, 13-15). Also, it was observed that these RPPs cannot individually activate the archaeal RPR, suggesting that heterodimerization is important for function. Additionally, NMR and yeast two-hybrid studies provided strong evidence for heterodimerization of POP5 with RPP30 and RPP21 with RPP29 (16). In fact, the Gopalan laboratory has exploited these findings and co-overexpressed the interacting archaeal RPPs in *Escherichia coli* and purified these proteins as binary complexes under native conditions (5, 15, 16).

Despite the recent advances in studying bacterial and archaeal RNase P, the exact interplay between the RNA and protein subunits in eukaryal RNase P remains elusive. So

far, native yeast and human RNase P have been purified to homogeneity and their subunit composition characterized. Human RNase P has ten RPPs associated with its RPR (H1 RNA) (17-19). The nomenclature of the protein subunits is based largely according to the molecular weight of each RPP: POP1, RPP40, RPP38, RPP30, RPP29, RPP25, RPP21, RPP20, POP5, and RPP14 (Table 1). Yeast RNase P has one RPR and nine subunits all of which share homologs with the human counterpart (2, 18). Despite having in hand the various recombinant subunits, it has not been easy to determine why eukaryotic RNase P requires nine to ten RPPs for biological function.

One approach to study the roles of human RPPs would be to biochemically reconstitute the human RPR (H1 RNA) with subsets of human RPPs and determine their effects (either alone or in combination) on RNA catalysis. In fact, such an undertaking revealed that human RPP21 and RPP29 facilitated H1 RNA-mediated pre-tRNA processing *in vitro* (17). The partial reconstitution of human RPR with RPP21 + RPP29 resulted in pre-tRNA cleavage but this activity was estimated to be less than 0.01% of that exhibited by a purified HeLa nuclear RNase P (20). Moreover, this activity could not be increased further upon addition of any of the remaining eight RPPs. Thus, it is unclear if the RNP complex consisting of H1 RNA + RPP21 + RPP29 is a true intermediate in the assembly towards the complete holoenzyme. Regardless, we have decided to adopt a slightly different approach to this daunting problem of reconstituting a 11-member RNP complex.

Given our recent successes with binary RPP complexes, our specific goal is to co-overexpress and purify human RPPs as binary complexes, with the expectation that such an approach can preserve the active conformations of human RPPs and thus facilitate successful *in vitro* reconstitution of human RNase P. Importantly, this should simplify the number of assembly paths and possible intermediates.

Having purified human RPPs as binary complexes (POP5•RPP30, RPP21•RPP29, RPP14•RPP30 and RPP20•RPP25) will also permit us to test them for their ability to heterologously reconstitute with the bacterial RPR; for these studies, we typically use M1 RNA, the RNA subunit of *E. coli* RNase P. These experiments will help identify if human RPPs promote bacterial RPR catalysis, a research goal motivated by the use of bacterial RNase P-derived customized ribozymes (M1GS) for selective and targeted degradation of viral and oncogenic mRNAs in mammalian cells. M1GS is a customized RNA in which the *E. coli* RPR (M1 RNA) is covalently tethered to a guide sequence (GS), which is designed to be complementary with the target RNA (21-23; Fig. 2). The guide sequence can be tailored to bind to any cellular RNA (e.g., oncogenic or viral mRNAs), and M1 RNA facilitates cleavage of the target mRNA once it is recognized and bound to the GS (21-23; Fig. 2). It remains unclear which human proteins assist M1GS to accomplish its task in human cells. Since it is conceivable that some human RPPs might act as cofactors for M1GS, we are also pursuing this research direction.

CHAPTER 2

METHODS

2.1 Protein overexpression

To co-overexpress human binary RPPs in *Escherichia coli* BL21 (DE3) cells, we used two compatible plasmids each encoding for one RPP gene: pET-15b (RPP30 or RPP20 or RPP21) and pLANT2b (RPP25 or RPP14 or POP5) (18). Note that pET-15b and pLANT2b confer resistance to carbenicillin and kanamycin, respectively. While *E. coli* BL21 (DE3) cells were transformed with the appropriate combination of plasmids (POP5•RPP30, RPP20•RPP25, RPP14•RPP30) to co-overexpress binary RPPs, *E. coli* BL21 (Rosetta) was used as the host to express RPP21 singly. Single transformants were picked to inoculate 6 ml of LB media containing 35 µg/ml kanamycin and 100 µg/ml carbenicillin (in those instances where two RPPs were being expressed from pET-15b and pLANT2b). The cultures were grown overnight and used as starter cultures to inoculate 500 ml of LB media supplemented with the appropriate antibiotics. The cells were grown at 37˚C and induced at Abs_{600} ~ 0.6-0.8 with isopropyl-β-D-thio-galactoside (IPTG). Human RPP20•RPP25 was induced with 1 mM IPTG for 3 h at 37˚C, while POP5•RPP30 and RPP14•RPP30 were induced with 1 mM IPTG at 25˚C for 17-19 h. Human RPP21 was induced with 1 mM IPTG, 100 mM $ZnCl_2$ at 25˚C for 17.5 h. These conditions were empirically identified to be the most optimal.

2.2 Purification of human binary RPP complexes

All purifications were performed on an AKTA Purifier FPLC system. All chromatographic columns were purchased from GE Healthcare. All purified RPP complexes were dialyzed against 25 mM Tris-HCl (pH 7.5), 400 mM ammonium acetate and 10 mM magnesium acetate (the RNase P assay buffer which we used), supplemented with 25% glycerol, and stored at -20°C until further use.

2.2.1 Human RPP20•RPP25

The frozen cell pellets from 125 ml of overexpressed culture were thawed and re-suspended in 25 ml of buffer S [25 mM Tris-HCl (pH 7.5), 1 M NaCl, 5 mM DTT, 0.1 mM PMSF, 1 mM EDTA]. Subsequently, the cells were lysed by sonication [4 sonication cycles, 1.5 min per cycle, program: 4 s on and 5 s off, amplitude: 80% (Sonics, VCX130)] and centrifuged at 11,000 g for 18 min at 4 ˚C to remove insoluble material and cell debris. The supernatant was treated with 0.05% (v/v) of polyethylenimine (PEI) to precipitate the nucleic acids. The PEI-precipitated nucleic acid was pelleted by centrifugation at 11,000 g for 18 min at 4˚C. The resulting supernatant was precipitated using 40% (w/v) ammonium sulfate and centrifuged at 11,000 g for 18 min at 4˚C. The precipitated pellet (containing RPP20•RPP25) was re-suspended in 10 ml of buffer A [25 mM Tris- HCl (pH 7.5), 25 mM NaCl, 5 mM DTT, 0.1 mM PMSF, 1 mM EDTA] and

dialyzed in buffer A for 3.5 h. The solution was filtered through a 0.45 µm filter and loaded on a 1-ml HiTrap SP-Sepharose column. The bound protein proteins were eluted with a linear (0.025-2 M) NaCl gradient. Human RPP20•RPP25 eluted between 0.68 and 0.74 M NaCl. Subsequently, the peak fractions (identified as such by SDS-PAGE and Coomassie blue staining) were pooled and supplemented with NaCl to a final concentration of 2 M NaCl and loaded on a 1-ml HiTrap phenyl-Sepharose column. Bound proteins were eluted using a reverse linear (2 -0.025 M) NaCl gradient. Human RPP20•RPP25 eluted at 0.08 M NaCl.

2.2.2 Human RPP14•RPP30

The frozen cell pellets from 125 ml of an overexpressed culture were thawed and re-suspended in 25 ml of buffer S (without EDTA). Subsequently, the cells were lysed by sonication [four sonication cycles, 1.5 min per cycle, program: 4 s on and 5 s off, amplitude: 80% (Sonics, VCX130)] and centrifuged at 11,000 g for 18 min at 4˚C to remove insoluble material and cell debris. The supernatant was treated with 0.1% (v/v) of polyethylenimine (PEI) to precipitate the nucleic acids, pelleted by centrifugation for 18 min at 4˚C. The resulting supernatant was precipitated using 40% (w/v) ammonium sulfate and centrifuged at 11,000 g for 18 min at 4˚C. The precipitated pellet (containing RPP14•RPP30) was re-suspended in 10 ml of buffer A and dialyzed for 16 h. The solution was filtered through a 0.45 µm filter and loaded on a 1-ml HiTrap SP-Sepharose

column. Human RPP14•RPP30 eluted between 1.6 and 1.7 M NaCl. Aliquots of eluted fractions were analyzed for presence of RPP14•RPP30 by SDS-PAGE followed by Coomassie blue staining.

2.2.3 Human POP5•RPP30

The purification protocol for human POP5•RPP30 was the same as the one employed for human RPP14•RPP30 with some exceptions. The protein complex eluted between 1 and 1.1 M NaCl after cation exchange (SP Sepharose) chromatography. The peak fractions were pooled and supplemented with NaCl to 2 M and loaded on a 1-ml HiTrap phenyl-Sepharose column. Bound proteins were eluted using a reverse linear (2 -0.025 M) NaCl gradient, and human POP5•RPP30 eluted at 0.025 M NaCl.

2.2.4 Human RPP21

The frozen cell pellets from 125 ml of an overexpressed culture were thawed and re-suspended in 25 ml of buffer N [25 mM Tris-HCl (pH 8), 0.5 M NaCl, 0.1 mM PMSF]. Subsequently, the cells were lysed by sonication [four sonication cycles, 1.5 min per cycle, program: 4 s on and 5 s off, amplitude: 80% (Sonics, VCX130)] and centrifuged at 11,000 g for 18 min at 4˚C to remove insoluble material and cell debris. The supernatant was filtered through a 0.45 µm filter and loaded on a HisTrap HP 1-ml (Nickel-

Sepharose) column. The bound protein was eluted using a linear (0-0.3 M) imidazole gradient, and human RPP21 eluted at 0.06 M imidazole. The peak fractions were pooled and supplemented with NaCl to 2 M and loaded on a 1-ml HiTrap phenyl-Sepharose column. The bound protein was eluted using a reverse linear (2 -0.025 M) NaCl gradient, and human RPP21 eluted at 1.9 M NaCl.

2.2.5 RNase P assays

Pre-tRNA processing assays were performed with tobacco chloroplast pre-tRNA^{Gly} as the substrate. For *in vitro* reconstitutions, RNase P was assayed in 25 mM Tris-HCl (pH 7.5), 400 mM ammonium acetate and 10 mM magnesium acetate, and incubated at 37°C. *E. coli* RPR (M1 RNA) was folded by incubation at 50°C for 50 min, 37°C for 10 min in water, and 37°C for 30 min in assay buffer. Reconstitution was initiated by incubating the folded *E. coli* RPR together with human RPPs (POP5•RPP30 or RPP14•RPP30) for 10 min at 37°C. After the holoenzyme was reconstituted (50 nM RPR and 1 μM of the specified binary RPP complex), the assay was initiated by adding pre-tRNA^{Gly} (250 nM, mixed with a trace amount of radiolabeled substrate), and incubated for 10 min. At the end of this incubation, RNase P reactions were quenched with an urea-phenol dye [8 M urea, 10% (v/v) phenol, 5 mM EDTA, 0.05% (w/v) xylene cyanol]. Reaction products were separated by denaturing polyacrylamide gel electrophoresis [8% (w/v) polyacrylamide gel, 8 M urea]. Subsequently, the products were visualized via phosphorimaging on

Typhoon (GE Healthcare) and quantitated by ImageQuant (GE Healthcare) to determine the extent of cleavage.

CHAPTER 3

RESULTS & DISCUSSION

We investigated different approaches to purify human RNase P proteins. Our overarching strategy though was to exploit the information on protein-protein interactions within the human RNP complex. This was motivated primarily by our own work with archaeal RPPs where we had discovered that function was dictated by heterodimerization (POP5•RPP30 and RPP21•RPP29) (5, 13). Moreover, yeast two-hybrid studies of human RNase P revealed the presence of strong protein-protein interactions between human POP5 and RPP30, RPP14 and RPP30, RPP20 and RPP25, RPP21 and RPP29 (2). These observations collectively provided the rationale to purify four different human RPP binary complexes (RPP14•RPP30, POP5•RPP30, RPP21•RPP29 and RPP20•RPP25).

The purification scheme of each protein complex was chosen to exploit the proteins' varying biochemical properties: size, charge, solubility difference, and hydrophobicity. PEI treatment, ammonium sulfate precipitation, cation-exchange and hydrophobic exchange chromatography were used to isolate the RPP pairs to homogeneity (Figs. 4-10). Indeed, three of the four complexes that we had set out to isolate were overexpressed and purified to homogeneity. These efforts were fruitful largely through empirical determination of different induction regimens to obtain soluble protein and examination of different chromatographic matrices to exploit the specific

biochemical features of the RPPs. It is important to point out that while affinity chromatography would have simplified the task of purifying these proteins, the presence of affinity tags (if unremoved) would have posed hurdles during assembly of this multi-protein RNP complex. Hence we invested significant time and effort to purify these binary RPPs as native complexes.

Although some preliminary efforts to reconstitute human RNase P RNA (H1 RNA) with these binary RPPs were not encouraging (Lai and Gopalan, unpublished results), we decided to pursue heterologous reconstitution with the bacterial RPR (M1 RNA from *E. coli*). We chose assay conditions that would allow for assessment of the beneficial effect of human binary RPPs on M1 RNA-mediated catalysis. When M1 RNA was reconstituted with human POP5•RPP30 and RPP14•RPP30, the holoenzyme exhibited multiple turnover at 37˚C with pre-tRNA^{Gly}. Based on the initial assay results, there was modest activation of bacterial RPR catalysis by POP5•RPP30 (Fig. 11).

While there is weak pre-tRNA processing from heterologous reconstitution, we will continue to optimize different conditions in order to maximize bacterial RPR catalysis in the presence of human RPPs. Altering divalent (Mg^{2+}) and monovalent (NH_4^+) ions would provide an array of conditions to examine if we could realize better activation of M1 RNA by human RPPs. Though Mg^{2+} is vital for M1 RNA folding and

catalysis, high concentrations of Mg^{2+} or NH_4^+could compromise and inhibit the interaction of RPPs with the M1 RNA. It is also possible that high concentrations of ammonium acetate might help dissociate non-specific protein-RNA interactions and increase the hydrophobic stacking effect of RPPs. Thus finding the optimal concentrations of divalent (Mg^{2+}) and monovalent (NH_4^+) ions in these assays will remain an important future goal.

The heterologous reconstitution assays with bacterial RPR shows a modest difference between POP5•RPP30 and RPP14•RPP30. POP5•RPP30 highly activated *Eco* RPR but RPP14•RPP30 showed minimal activation of *Eco* RPR. Phylogenetic analyses suggest human POP5 and RPP14 share evolutionary ancestry and a paralogous relationship (Fig. 12) (24). There was an activation difference upon reconstituting these related binary complexes with M1 RNA, which imply that RPP14 does not behave in an identical fashion as POP5; in fact, it is already established that RPP14 (unlike POP5) does not directly interact with H1 RNA (20).

Based on our results, there is a possibility that RPP14•RPP30 and POP5•RPP30 might not be sufficient for M1 RNA or H1 RNA activation. Although we have co-overexpressed and purified human RPP subunits as binary complexes, we have also made attempts at overexpressing and purifying other human RPPs as single subunits. Human RPP21•RPP29 was not co-overexpressed and purified successfully, but we were able to

overexpress and purify human RPP21 as a single RPP (Fig. 13). The weak overexpression of RPP21•RPP29 could be attributed to their codon usage being suboptimal in *E. coli*. Since there is evidence of human RPP21 weakly activating H1 RNA (17, 20), we could use RPP21 in tandem with the other purified human RPP binary complexes for heterologous reconstitution.

Since our long-term goal is to reconstitute the human RNase P holoenzyme *in* vitro, we would need to complete purification of the entire suite of RPPs and then test different combinations with H1 RNA. POP1, the largest of the RPPs (~100 kDa), might well prove critical in providing a scaffold for RNase P assembly. If so, we will not be able to move forward without purifying it. Genetic studies suggest that POP1 is an essential RPP subunit in yeast RNase P catalysis. Mutagenizing POP1 resulted in loss of assembly and *in vivo* function of yeast RNase P (26). Our initial attempts to purify human POP1 revealed that it is subject to proteolysis (unpublished observations).

It is possible that not all RPPs might lend themselves to the binary RPP purification approach. Ultimately, the challenging goal of reconstituting human RNase P might depend on a combinatorial approach wherein some RPPs are isolated as binary complexes while others as individual proteins. This thesis provides a foundation to make advances in this direction.

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FIGURES & TABLES

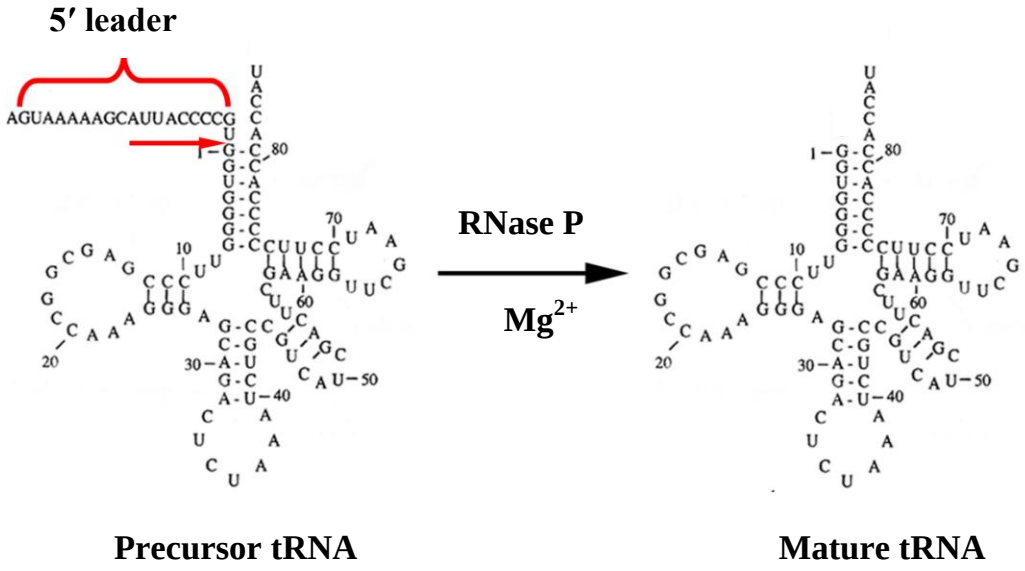

Figure 1. 5′-Maturation of tRNAs.

RNase P aids in tRNA biogenesis by cleaving the 5′ leader of precursor-tRNA. It requires Mg^{2+} as an essential cofactor.

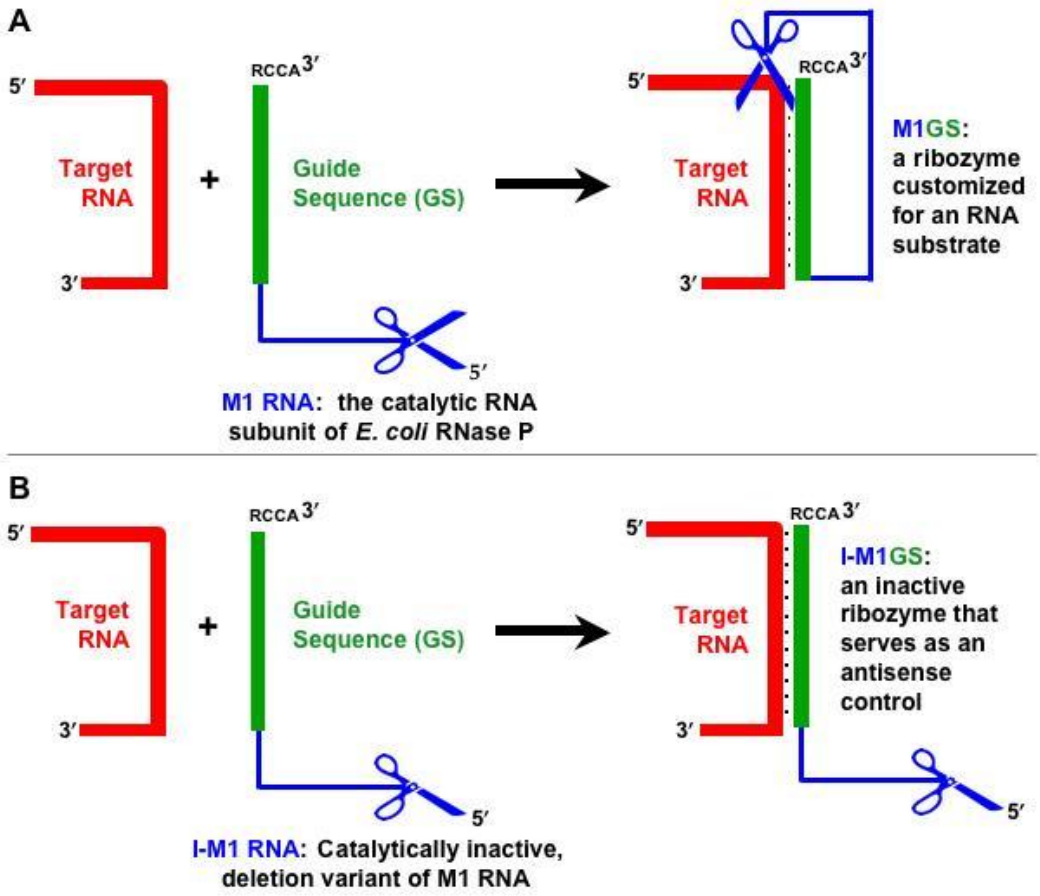

Figure 2. Use of customized RNase P-based ribozymes for targeted degradation of cellular RNAs.

(A) M1GS uses the guide sequence (GS) to bind to the target mRNA by complementary base pairing thereby enabling M1 RNA to cleave the target mRNA. (B) I-M1GS is catalytically inactive due to point mutations in M1 RNA, and as a result, there is no cleavage activity. I-M1 GS is used as a negative control to correct for antisense effects. This figure is modified from reference 20.

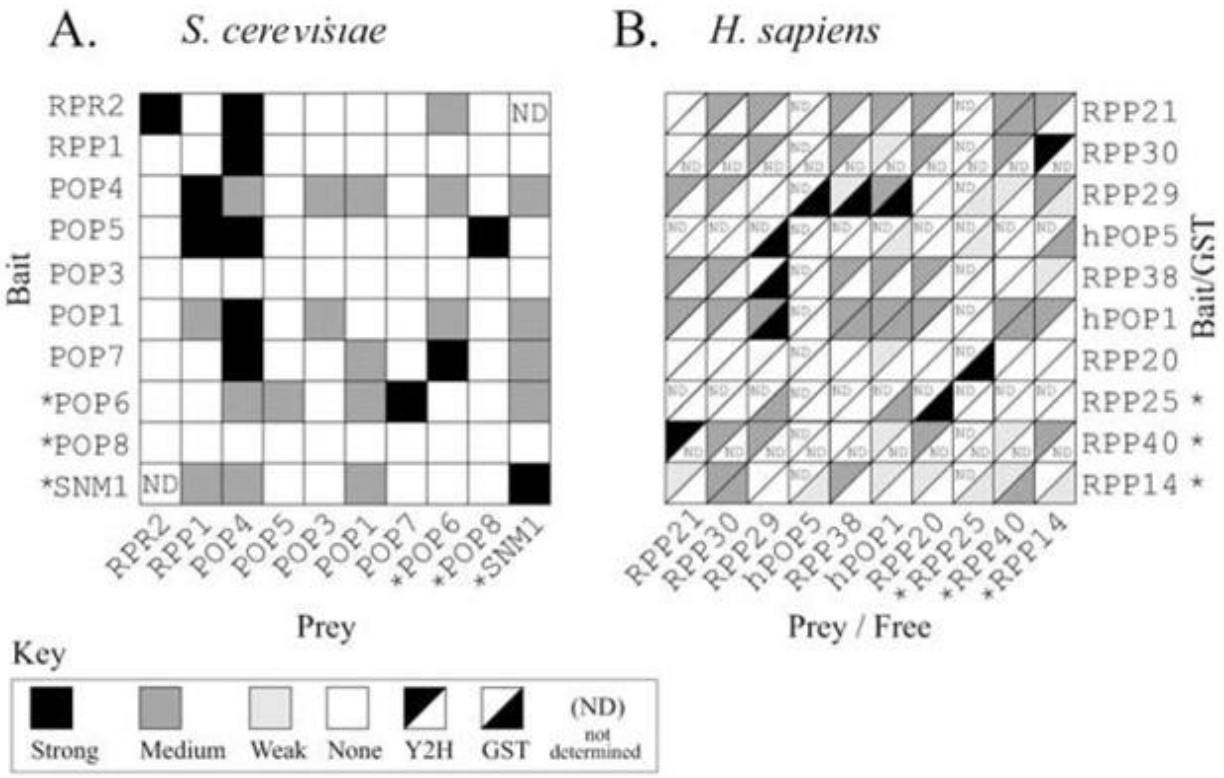

Figure 3. Protein-protein interactions within yeast (A) and human (B) RPPs using yeast two-hybrid (Y2H) genetic studies and glutathione-S-transferase (GST)-based pull-down experiments. This figure is reproduced from reference 2.

Table 1

Composition of RNase P in the three domains of life

Bacteria (*Eco)*	Archaea (*Mma)*	Eukarya (*Hsa)*
RPR	RPR	RPR
1 RNA	1 RNA	1 RNA
(377 nt)	(250 nt)	(339 nt)
RPP	RPPs	RPPs
1 RPP	4-5 RPPs	10 RPPs
C5 (14)		POP1 (115)
		RPP40 (40)
	L7Ae (12)	**RPP38 (32)**
	RPP30 (26)	**RPP30 (29)**
	RPP29 (11)	**RPP29 (25)**
		RPP25 (21)
	POP5 (15)	**POP5 (19)**
	RPP21 (13)	**RPP21(18)**
		RPP20(16)
		RPP14 (14)

RPPs highlighted in bold font indicate homologs. The length of the RPR and the molecular weight of each RPP are provided in parentheses.

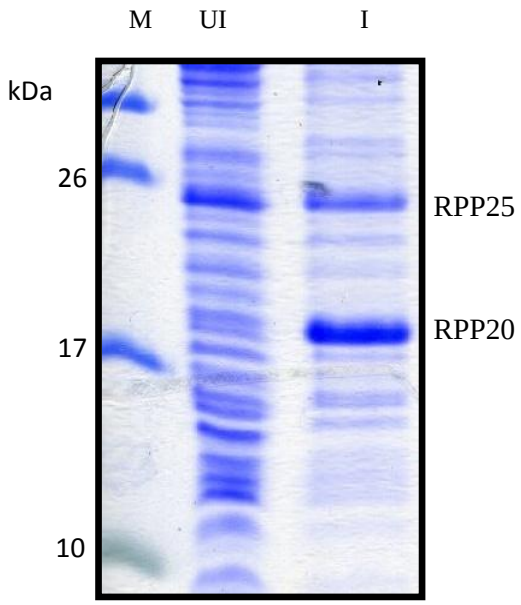

Figure 4. SDS-PAGE analysis to demonstrate co-overexpression of recombinant human RPP20•RPP25.

UI and I indicate the crude extracts from un-induced and induced cultures of *E. coli* BL21 (DE3) cells, respectively, that co-overexpress RPP20 and RPP25 from pET-based overexpression plasmids. M denotes a reference size marker.

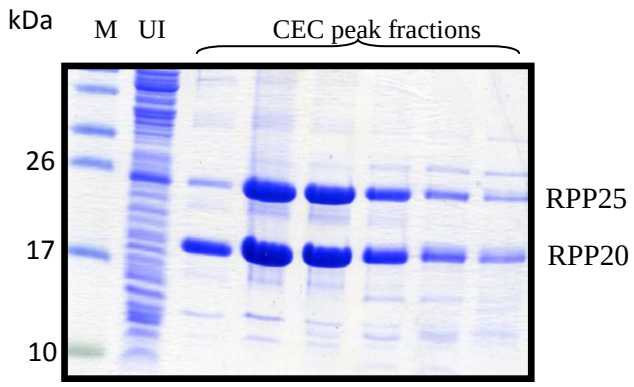

Figure 5. SDS- PAGE analysis to demonstrate the purification of recombinant human RPP20•RPP25.

UI indicate the crude extracts from un-induced culture in *E. coli* BL21 (DE3) cells. CEC peak fractions correspond to those obtained after cation exchange chromatography. M denotes a reference size marker.

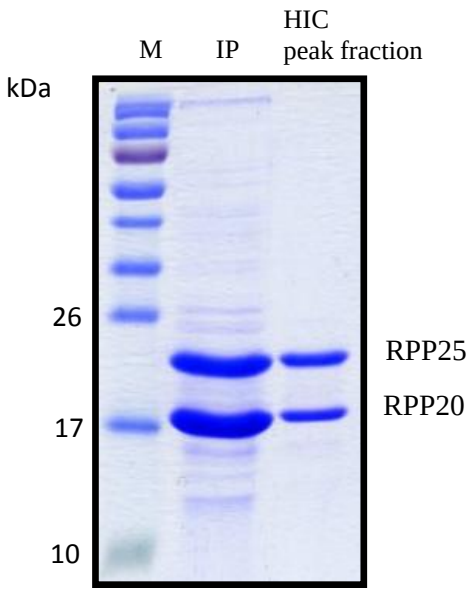

Figure 6. SDS-PAGE analysis to demonstrate purification of recombinant human RPP20•RPP25.

IP comprises of pooled fractions from cation exchange chromatography, and the HIC peak fraction corresponds to those obtained after hydrophobic interaction chromatography. M denotes a reference size marker.

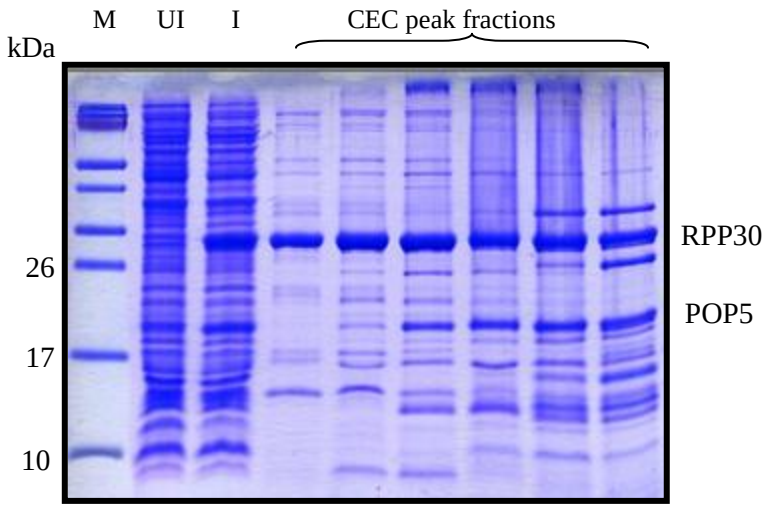

Figure 7. SDS-PAGE analysis to demonstrate purification of recombinant human POP5•RPP30.

UI and I indicate the crude extracts from un-induced and induced cultures of *E. coli* BL21 (DE3) cells, respectively, that co-overexpress POP5 and RPP30 from a pET-based overexpression plasmid. CEC peak fractions correspond to those obtained after cation exchange chromatography. M denotes a reference size marker.

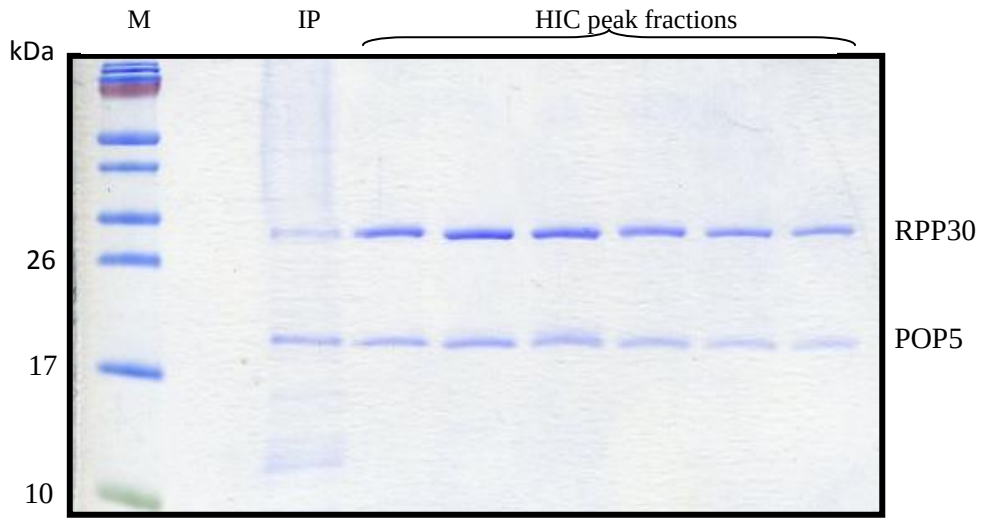

Figure 8. SDS-PAGE analysis to demonstrate purification of recombinant human POP5•RPP30.

IP is the input from the combined CEC peak fractions. The HIC peak fractions correspond to those obtained after hydrophobic interaction chromatography. M denotes a reference size marker.

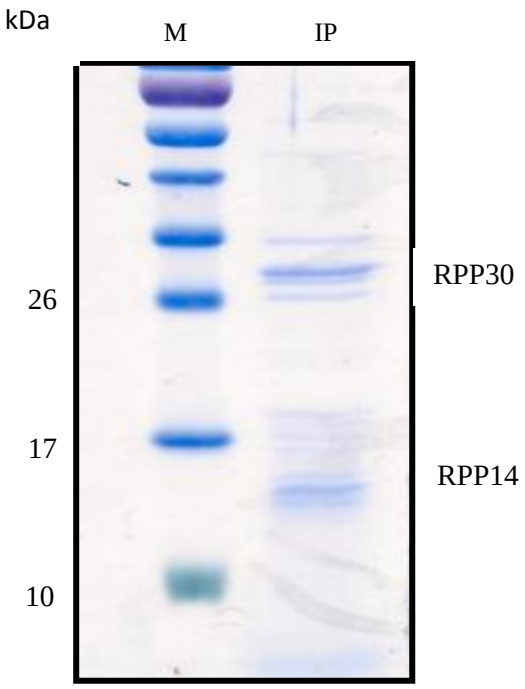

Figure 9. SDS-PAGE analysis to demonstrate purification of recombinant human RPP14•RPP30.

IP is the solubilized cell pellet after ammonium sulfate precipitation. M denotes a reference size marker.

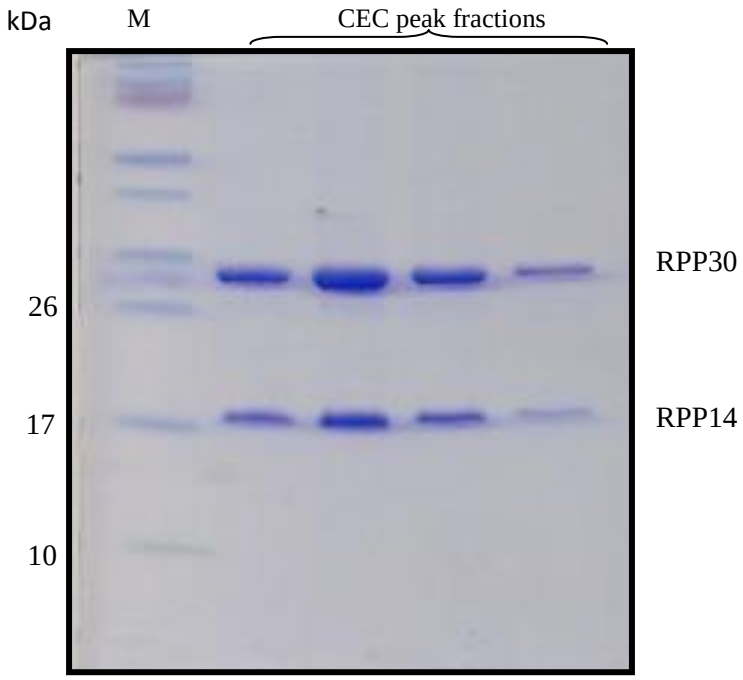

Figure 10. SDS-PAGE analysis to demonstrate purification of recombinant human RPP14•RPP30.

CEC peak fractions correspond to those obtained after cation exchange chromatography. M denotes a reference size marker.

Eco RPR (50 nM)	+	+	+	-	+	-	-
Eco RPP (1 µM)	-	-	+	-	-	-	-
Human POP5•RPP30 (1 µM)	-	+	-	-	-	-	+
Human RPP14•RPP30 (1 µM)	-	-	-	-	+	+	-

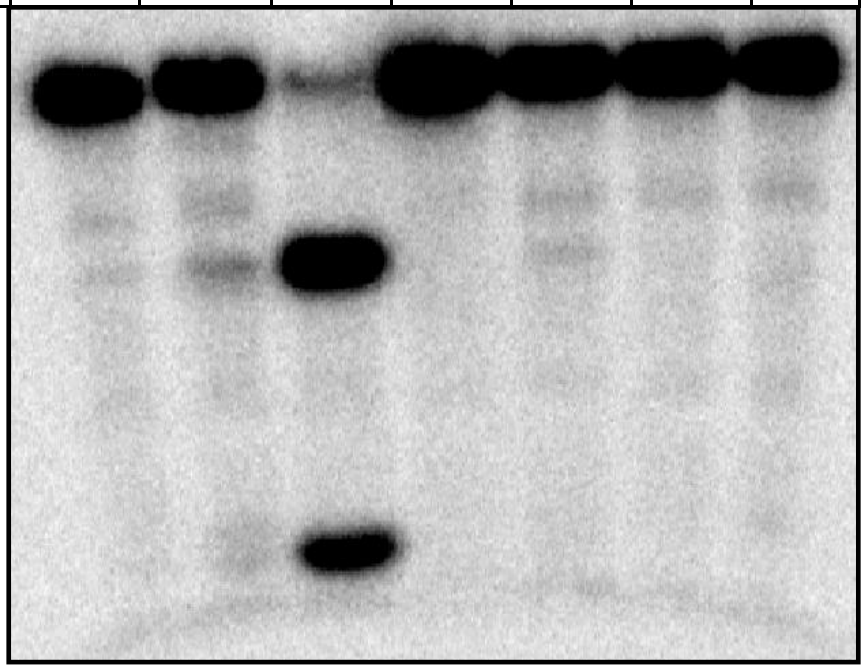

Figure 11. Heterologous reconstitution of human binary RPPs (POP5•RPP30 or RPP14•RPP30) with *Eco* RPR.

Results of the heterologous reconstitution of *Eco* RPR (M1 RNA) and human RPPs. RNase P was assayed in 25 mM Tris-HCl (pH 7.5), 400 mM ammonium acetate, 10 mM magnesium acetate, 250 nM pre-tRNA^{Gly}, and incubated at 37°C for 10 min.

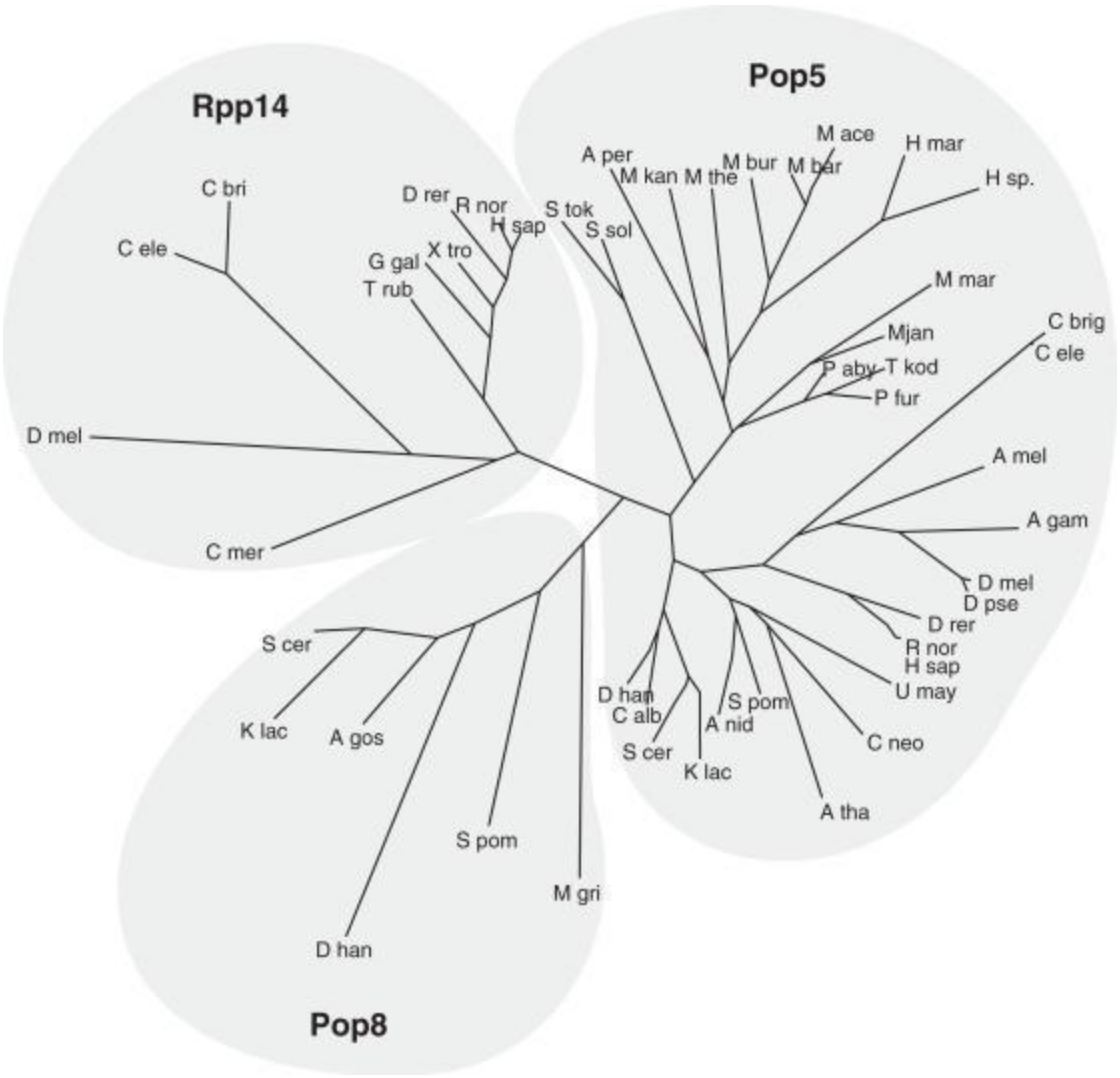

Figure 12. A phylogenetic tree showing RPP relationships within eukaryal POP5, RPP14, and POP8.

A phylogenetic tree was constructed to show the relationship among different archaeal and eukaryal RNase P proteins (RPP14 and POP5). Human RPP14 and POP5 share a paralogous relationship. This figure is reproduced from reference 25.

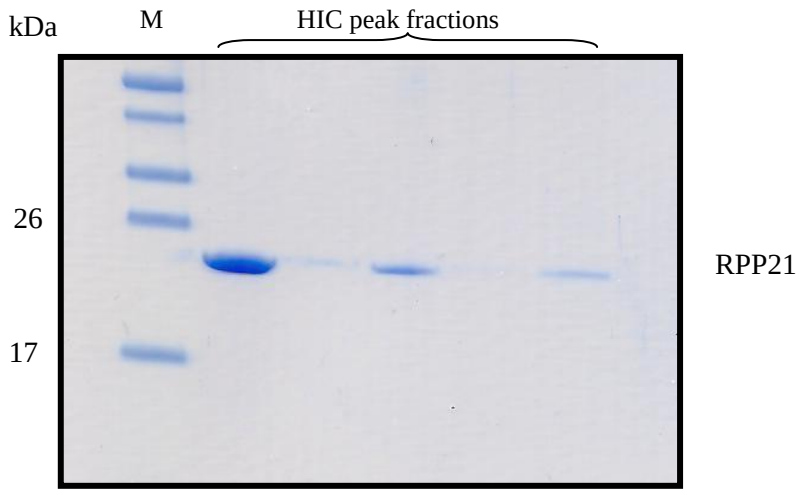

Figure 13. SDS-PAGE analysis to demonstrate purification of recombinant human RPP21.

HIC peak fractions correspond to those obtained after hydrophobic interaction chromatography. M denotes a reference size marker.